Gastrointestinal modifications and bioavailability of brown seaweed phlorotannins and effects on inflammatory markers.

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Short title: The bioavailability of seaweed phlorotannins

Keywords: Polyphenols, phlorotannins, brown seaweed, bioavailability, metabolism, human

Abstract:

Brown seaweeds such as *Ascophyllum nodosum* are a rich source of phlorotannins (oligomers and polymers of phloroglucinol units), a class of polyphenols that are unique to Phaeophyceae. At present there is no information on the bioavailability of seaweed polyphenols and limited evidence on their bioactivity *in vivo*. Consequently we investigated the gastrointestinal modifications *in vitro* of seaweed phlorotannins from *Ascophyllum nodosum* and their bioavailability and effect on inflammatory markers in healthy participants. *In vitro*, some phlorotannin oligomers were identified after digestion and colonic fermentation. In addition 7 metabolites corresponding to *in vitro* absorbed metabolites were identified. Urine and plasma samples contained a variety of metabolites attributed to both un-conjugated and conjugated metabolites (glucuronides and/or sulfates). In both urine and plasma, the majority of the metabolites were found in samples collected at late time points (6-24 h), suggesting colonic metabolism of high molecular weight phlorotannins, with three phlorotannin oligomers (hydroxytrifuhalol A, 7-hydroxyeckol, C-O-C dimer of phloroglucinol) identified in urine samples. A significant increase of the cytokine IL-8 was also observed. Our study shows for the first time that seaweed phlorotannins are metabolized and absorbed, predominantly in the large intestine, and there is a large inter-individual variation in their metabolic profile. Three phlorotannin oligomers present in the capsule are excreted in urine. Our study is the first investigation of the metabolism and bioavailability of seaweed phlorotannins and the role of colonic biotransformation. In addition IL-8 is a possible target for phlorotannin bioactivity.

Introduction: There has been increasing interest in the past few years on the bioactive compounds present in seaweeds [1; 2; 3]. Traditionally, seaweeds are consumed as a food product in Asian countries and are increasingly used worldwide as ingredients for industrial applications. In Japan, over 20 species of red, green, and brown algae (seaweed) are included in meals [4], and daily seaweed consumption per person has remained relatively consistent over the last 40 years, in the range of 1.50 to 3.65 kg/person/year as reported by a range of studies [5; 6; 7]. Seaweeds are a rich source of polyphenolic compounds [8], and polyphenols extracted from algae [9; 10] show some similarities to those found in land plants [9; 10; 11]. Thus the main polyphenols found in brown seaweeds are phlorotannins [12; 13; 14; 15], a type of phenolic compound only found in brown seaweeds [16]. Brown seaweed phlorotannins are oligomers and polymers of phloroglucinol units, and their oligomer and polymer molecular weights can greatly vary, from 126 Da to 650 kDa [3], comprising up to 15 % of the plant dried weight [11]. It has been reported that the consumption of brown algae is on average 1.342 kg/person/year, containing 66.652 g of phlorotannins/person/year and 183 mg/person/day [4] [4]. Phlorotannins are classified according to the type of linkages between phloroglucinol units into four main groups: eckols (with dibenzodioxin linkages), fucols (with a phenyl linkage), fuhalols and phloroethols (with ether linkages), and fucophloroethols (with ether and phenyl linkages) [16]. Phlorotannins are being increasingly investigated for their vast array of bioactivities [10; 17; 18] such as antioxidant [19; 20; 21; 22; 23; 24], anti-inflammatory [20; 25; 26], antibacterial [27; 28], anticancer [29; 30; 31; 32; 33], and antidiabetic [29; 34; 35], showing promising potential to further develop seaweed-derived products rich in bioactive components with commercial potential for food and pharma applications [36].

Bioavailability is a critical factor influencing *in vivo* biological activity of polyphenols and we have reasonable understanding of the bioavailability of polyphenols from fruits and vegetables, and some of the mechanisms by which they exert beneficial effects *in vivo* have been determined [37]. Their ability to act as effective bioactive molecules *in vivo* is dependent on the extent of their biotransformation [24] and conjugation during absorption from the gastrointestinal (GI) tract, in the liver and finally in cells [37]. Consequently, consideration must be given to the way polyphenols are absorbed and metabolised during gastrointestinal digestion and colonic fermentation and how this may impact on bioactivity [38]. It is noteworthy that there is no information on the bioavailability of seaweed phlorotannins and this is a limitation to understanding their bioactivity and mechanism of action *in vivo*. In the absence of specific data regarding phlorotannin absorption and bioavailability, it is useful to consider the absorption and metabolism of other polyphenols as a guide [37]. In general, after ingestion of a polyphenol-

rich diet, their protective effects *in vivo* are determined by measuring a range of suitable biomarkers, and correlate with the absorption of polyphenols from the gut and their circulation/excretion [38]. Polyphenols can be extensively conjugated to form glucuronide, sulphate and methyl group in the gut mucosa and inner tissues [37; 39], and absorption occurrs in the small intestine [37]. Polyphenols unabsorbed in the upper gastrointestinal tract or re-excreted in the bile, are extensively metabolised by colonic microflora into a wide range of low molecular weight phenolic acids [40]. The aim of this study was to elucidate the gastrointestinal modifications of seaweed phlorotannins, and the effects on their metabolism and bioavailability. A food grade seaweed polyphenol extract (SPE) rich in phlorotannins (from the brown seaweed *Ascophyllum nodosum)* was subjected to *in vitro* gastrointestinal digestion and fermentation to examine the gastrointestinal modifications occurring in the upper- and lower- GI tract. Furthermore, the absorption and metabolism of polyphenols in healthy subjects was investigated, after oral ingestion of a SPE capsule containing 101.89 mg of polyphenols. This amount represents an intake lower than the average daily intake of seaweed polyphenols in the Asian diet, and it is not expected to exert any cytotoxic effect [41]. The impact of absorption and gastrointestinal modifications on phlorotannins anti-inflammatory potential is explored.

Experimental methods

Seaweed material. Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of Lewis, Scotland in March 2011. The seaweed biomass was harvested by hand, cleaned and then shipped refrigerated to the processing facility in France where it was immediately chopped and frozen.

Preparation of food-grade SPE and capsule. A novel SPE from *Ascophyllum nodosum* was produced by CEVA (France) using a solvent based extraction system that was specifically developed for this study and for use with either fresh or frozen *Ascophyllum nodosum*. The solvent used was a 60:40 ethanol:water mixture which allowed for the water content of the seaweed itself. The extraction was carried out over 5 hours using constant stirring and at all times protected from light. A solvent:seaweed ratio of 3:1 was used. The mixture was filtered to remove the supernatant and subsequently the alcohol was removed using a rotary evaporator. A hydrometer was used to check that all the alcohol had been removed. The final extract was recovered by centrifugation and further filtration before freeze drying.

Approximately half of the produced extract (basic extract) was then fractionated using tangential flow ultra filtration to produce further extracts of varying molecular weight range and with varying polyphenol content. A blended SPE was formulated (Table 1) using 175 mg of basic extract and 50 mg of high molecular weight (HMW) fraction (>10 kDa cut off) for use in the current study. Maltodextrin (175 mg) was added to the capsule formulation as an excipient. This was done in order to maximise the polyphenol content (>100 mg per day) but also to minimise the level of iodine to within accepted regulatory guidelines (< 500 µg per day). Blending was carried out at the food grade CEVA facilities in France. 400 mg doses of the SPE *Ascophyllum* blend were packed into white, opaque, vegetarian capsules by Irish Seaweeds, Belfast, UK and used for the clinical study. The food grade seaweed capsule was characterized by NP-HPLC and LC-MS analysis. Phlorotannins were quantified using the Folin-Ciocalteau Method [42] using phloroglucinol as the standard [8].

Simulated Gastro-Intestinal Digestion and Fermentation: The Gastro-Intestinal Digestion procedure was adapted from Mills *et al.* (2008) and McDougall *et al.* (2005). This method consists of two sequential stages: gastric digestion and small intestinal digestion followed by dialysis. 10 g of SPE was dissolved in 30 ml acidified water (pH=2) and pepsin (320 U/ml) was added. Samples were incubated at 37 °C for 2 h on a shaker covered with foil to protected

from light. 5 ml aliquots (G) were removed. The pH was adjusted to 7.5 by adding few drops of 6 M NaOH, and pancreatin (4 mg/ml) and bile extracts (25 mg/ml) were added. The samples were incubated at 37 °C for 2 h on a shaker. 5 ml aliquots (SI) were removed. Samples were transferred into the dialysis tubing (100-500 Da, cut-off, 1.8 ml/cm, Spectra/Por, Biotech) and dialysed overnight at 4 °C against water (4 L) to remove low molecular weight digests. 5 ml aliquots of dialysis solution (D1) were removed. The dialysis fluid was changed and dialysis continued for additional 2 h. 5 ml aliquots of second dialysis solution (D2) were removed. Samples (SI+D) were freeze-dried and subjected to colonic fermentation (Batch Culture): The method was adapted from Tzounis *et al*. (2008). Batch-culture fermentation vessels (300 ml; one vessel per treatment) were autoclaved and filled with 135 ml sterilized basal medium. Medium was stirred and gassed overnight with O2-free N2. Before addition of SI+D digested extracts equivalent to 1.5 g of undigested extracts, the temperature inside the vessels was set to 37 °C by a circulating water bath and the pH was controlled at 6.8 by an Electrolab pH controller, in order to mimic conditions in the distal region of the human large intestine (anaerobic; 37 °C; pH 6.8). Vessels were inoculated with 15 ml faecal slurry (1:10, w/v) and batch cultures were run for 24 h 7 ml samples were collected at five time points (0, 2, 4, 8 and 24 h), centrifuged at 13,000 rpm at 4 °C for 10 min and the supernatants were kept. All the samples collected during the digestion and fermentation procedure were stored at -80 °C until LC-MS analysis.

Study design: This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the University of Reading Ethics Committee before initiation of the study. Written informed consent was obtained from all participants. Exclusion criteria for subjects were: smokers, BMI < 18 or > 30, abnormal liver function and haematology, alcohol intake of > 21 units/wk, gastrointestinal disease or chronic gastrointestinal disorders, consumption of antibiotics in previous 3 months before study, females who were pregnant or intending to become pregnant. Potentially suitable participants underwent a screening process and individuals with blood pressure > 150/90 mmHg; Hb > 125 g/l for men and > 110 g/l for women; g-glutamyl transferase > 1·3 mkat/l or cholesterol > 6·5 mmol/l were excluded from the study. In total 24 volunteers were recruited - 12 females (6 aged 18-30 years and 6 aged 30-65 years) and 12 males (6 aged 18-30 years and 6 aged 30-65 years). Participants were asked to follow a low phenolic diet for 1 day prior to the study day (devoid of tea, coffee, fruit, vegetables, alcoholic beverages, cocoa, whole- grain and seaweed-containing products). On the study day, the

subjects were cannulated and a baseline blood sample was taken. Participants were asked to consume one SPE capsule (400 mg) containing 101.89 mg of polyphenols. Blood samples were collected at 0, 1, 2, 3, 4, 6, 8 and 24 hours after ingestion of the SPE capsule, and urine samples were collected at baseline, 0-8 and 8-24 h after the ingestion. During the day, participants were provided with a lunch and dinner of low phenolic content. The study is registered at clinicaltrials.gov (study ID: NCT02496806).

Sample collection and storage: One aliquot of blood was collected in heparin tubes and cultured immediately (whole blood culture for cytokine analysis). One aliquot of blood was collected in EDTA tubes and centrifuged at 3000 rpm for 15 min at 4˚C. The plasma was separated and 1 mg/ml ascorbic acid was added as preservative. Aliquots were stored at -80 ˚C until analysis. Total volume of collected urine was recorded and aliquots were stored at -80 ˚C until analysis.

Plasma sample processing for metabolite analysis: Plasma samples were prepared following a procedure similar to the one described by Ottaviani *et al*. [43]. 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 450 µl of plasma, then 50 µl of 1.2 M acetic acid were added and samples were mixed. Samples were analysed with and without enzymatic treatment (37 °C, 40 min) in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *Helix pomatia* (Type H-1). 1 ml of 100 % methanol acidified with 0.5 % acetic acid was added and samples were centrifuged for 15 min at 16,100 x g at 4 °C and supernatants were collected. This step was repeated 3 times (last time with 50 % methanol acidified with 0.5 % acetic acid) and the supernatants were dried using a Speedvac. The pellets were dissolved with 125 µl of mobile phase and transferred to vials for RP-HPLC analysis.

Urine sample processing for metabolite analysis: Urine samples were prepared following a procedure similar to the one described by Ottaviani *et al*. [43]. Briefly, 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 250 µl urine. Samples were analysed with and without enzymatic treatment (37 °C, 40 min), in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *H. pomatia* (Type H-1). 1 ml of 100 % methanol acidified with 0.5 % acetic acid was added, samples were mixed and centrifuged for 15 min at 16.100 g (rcf) at 4 °C, supernatants were transferred to a new tube and dried on a Speedvac. Dried samples were re-suspended on 125 µl of mobile phase, completely dissolved, centrifuged and transferred to vials for RP-HPLC analysis and LC-MS analysis.

NP-HPLC analysis: The phlorotannins in the food-grade SPE used to produce the capsule were analyzed by normal phase HPLC analysis [8] using an HPLC 1100 series equipped with LiChrospher Si60-5 column (250 mm × 4.0 mm ID, 5 µm particle size from HICHROM (LISP60-5-250AF), fitted with a guard column LiChrospher Si60-5 from HICHROM (LISP60-5-10C5). The mobile phase contained A: 82 % dichloromethane + 2 % methanol + 2 % acetic acid in water and B: 96 % methanol + 2 % acetic acid in water and was pumped through the column at 1 ml/min. 10 µl of samples were injected and analyzed by the gradient program which were (min/%B): 0/0, 30/17.6,45/30.7, 50/87.8, 60/87.8, 80/0, 105/0 for detection of all compounds. The compounds were detected at a wavelength of 268 nm. All data were analyzed by ChemStation software. The phloroglucinol standard was injected at 0.1-100 µg/ml and phlorotannins in the capsules were analysed as phloroglucinol equivalents.

RP-HPLC analysis: The analysis of plasma and urine samples was carried out with a Hewlett-Packard 1100 series liquid chromatograph (Hewlett-Packard, Palo Alto, CA) as previously described [44]. Samples were analyzed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 x 250 mm) with 4 µm particle size. The temperature of the column was maintained at 30 ºC. The mobile phases consisted of a mixture of aqueous methanol 5 % in 0.1 % hydrochloric acid 5 M (A) and a mixture of aqueous acetonitrile 50 in 0.1 % hydrochloric acid 5 M (B) and were pumped through the column at 0.7 ml/min. The following gradient system was used (min / % B): 0/5, 5/5, 40/50, 55/100, 59,9/100, 60/5, with 10 min post-run for both compound and metabolite detections. The eluent was monitored by photodiode array detection at 280 nm and spectra of products obtained over the 200–600 nm range. Peaks were characterized by their retention time and spectra characteristics. A calibration curve of phloroglucinol was constructed using authentic standards (0.1–100 µg/ml) and in each case were found to be linear with correlation coefficients of 0.995. Metabolites were quantified as phloroglucinol equivalents.

LC-MS analysis: LC-MS analysis was conducted to analyse the food grade seaweed capsule, the urine samples and the digested materials, and was carried out in the negative ion mode using LC-MS/MS utilizing electrospray ionisation (ESI) as previously described [45]. Characterization was achieved using LC-MS/MS utilizing electrospray ionisation (ESI). This consisted of an Agilent 1200 HPLC system equipped with a binary pump, degasser, auto-sampler, thermostat, column heater, photodiode array detector and an Agilent 1100 Series

LC/MSD Mass Trap Spectrometer. Separation of samples was achieved using a Zorbax SB C18 column (2.1 x 100 mm; 1.8 µm) (Agilent) and HPLC conditions were as follows: injection volume: 1 µL; column temperature: 25 °C; binary mobile system: (A) 0.1 % aqueous formic acid and (B) 0.1 % of formic acid in acetonitrile; flow rate: 0.2 mL/min. A series of linear gradients were used for separation (min/%B): 0/10, 3/10, 15/40, 40/70, 50/70, 65/10. Mass spectrometry was performed in the negative ion mode (scan range, m/z 100-1000 Da; source temperature, 350 °C). All solvents used were LC-MS grade.

Cytokine production: Blood samples collected during the clinical intervention (baseline, 1 h, 2 h, 4 h, 6 h, 8 h) into heparin tubes, were immediately cultured as follows: 500 µl blood aliquots were mixed with 500 µl of RPMI media containing antibiotics on a 24 well plate and LPS (1 µg/ml) or vehicle (control group) were added before incubation at 37 °C for 24 h. At the end of the culture period, samples were centrifuged at 2000 x g for 5min and the supernatants were collected and kept at -20 °C until analysis for inflammatory cytokine levels. The supernatants were collected and stored at -20 °C. Cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in the supernatants were measured with Luminex xMAP Technology using commercially available Fluorokine MAP kits (R&D systems) and data analyzed on the xPONENT software. Final data are presented as the difference between LPS-treated and un-stimulated control.

Statistical analysis: The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc t-test using GraphPad InStat version 5 (GraphPad Software, San Diego, CA, USA). Significant changes are indicated as $P < 0.05$.

Results

SPE characterization: The chromatogram (Figure 1) illustrates the trace obtained by NP-HPLC with diode array detection after injecting a water solution of the SPE. The chromatogram shows a number of peaks (20-70 min) representing different high molecular weight phlorotannins, the characteristic phenolics in brown seaweeds. Longer phlorotannin polymers, which consisted of more hydroxy groups, resulted in tighter attachment to the column material. Consequently, shorter compounds were released earlier than longer compounds. Owing to a lack of phlorotannin standards and the complexity of the oligomeric and polymeric forms, the calibration curve of phloroglucinol was used to quantify all the phlorotannins contained in the SPE as phloroglucinol equivalents. The SPE comprised a wide range of molecular weight of phloroglucinol derivatives with a total phlorotannin concentration of 312µg/mg quantified as phloroglucinol equivalents. Further characterization of the SPE was achieved with LC-MS/MS (Figure 2) utilizing electrospray ionisation (ESI). The data were collected in a non-targeted fashion, by acquiring full spectrum data in negative ion mode from m/z 100 to 1000. The data were then analysed by searching for the theoretical mono-isotopic masses corresponding to possible phlorotanninoligomers and the presence of ions (1-6) which could correspond to phlorotannins (Figure 2). The ion 1 with [M-H]- at m/z 405 corresponded to the trimer hydroxytrifuhalol A, whereas the MS2 fragment -387 corresponded to the loss of one molecule of water (-18), a characteristic pattern of phlorotannin fragmentation. Compound 2 ([M-H]- at m/z 497) was considered to be a phlorotannin tetramer composed of 4 phloroglucinol units, such as tetrafucol or fucodiphlorethol and also in this case the fragment -479 corresponds to the loss of a molecule of water (-18), whereas the fragment -353 corresponds to the loss of water (-18) and a phloroglucinol unit (-126), in accordance with analytical profiles recently described in positive ion mode by Wang *et al*. [46] and by Ferreres *et al*. [47]. The ion 3 has a [M-H]- at m/z 247 corresponding to a C-O-C dimer of phloroglucinol as previously indicated by Nwosu *et al* [29]. The ion 4 (387) corresponds to the trimer 7-hydroxyeckol, and we observe the presence of a fragment at -369 deriving from the loss of one molecule of water. Isomers 5 and 6 with [M-H]- at m/z 249 were also observed, which can correspond to the dimers diphlorethol and difucol.

***In vitro* digestion and characterisation:** Due to the lack of commercially available standards for phlorotannins and the complexity of the oligomers and polymers in the extract, the analysis of phlorotannins and their metabolites is challenging and requires a combination of approaches. Similarly to other polyphenol classes, phlorotannins may undergo extensive modification by

phase I and phase II enzymes and the colonic microbiota during their transit through the gastrointestinal tract [37], and the implication of such metabolic modifications on the bioactivity of phlorotannins has not been investigated yet. Consequently we subjected the SPE to *in vitro* digestive and fermentative processes. An *in vitro* gastric and ileal digestion and colon microbial fermentation of the SPE was initially conducted, followed by dialysis to simulate absorption into the circulation. The MS spectra and fragmentations of the compounds detected in the samples were studied (Figure 3). After *in vitro* digestion and fermentation procedures, the samples were analysed by LC-MS/MS utilizing electrospray ionisation (ESI) in negative ion mode as previously described, searching for the theoretical mono-isotopic masses corresponding to the low molecular weight phlorotannins previously identified in the capsule (Figure 2). We were able to identify molecular ions and fragments corresponding to hydroxytrifuhalol A (405), the C-O-C dimer of phloroglucinol (247), the dimer diphlorethol/ difucol (249) and 7-hydroxyeckol (387), also found after colonic fermentation. In addition, in digested and fermented samples subjected to dialysis to mimic absorption into the circulation, we reported the presence of 7 compounds (DM1 to DM7) corresponding to *in vitro* absorbed metabolites.

Plasma and urine analysis: The food grade SPE was given to healthy subjects (Figure 4) in the form of a capsule (400 mg) containing 101.89 mg of polyphenols, and blood and urines were analysed for phlorotannin metabolites. HPLC-DAD analysis of the plasma (Figure 5) and urine (Figure 6) samples with and without glucuronidase/sulfatase treatment showed the presence of a variety of metabolites absent in the baselines (before the seaweed capsule ingestion) in samples from 15 volunteers out of 24. In plasma, the total level of phlorotannins and their metabolites ranges from 0.011 to 7.757 µg/ml, and in urine from 0.15 to 33.52 are excreted. Some metabolite peaks were present in samples with and without enzymatic treatment, and therefore could be assigned to un-conjugated metabolites. Some other metabolite peaks were present only in samples without enzymatic treatment or were only appearing in samples enzymatically treated, and were attributed to conjugated forms (glucuronides and/or sulfates) and their enzymatically released un-conjugated forms. In urine, some metabolites were found in samples collected at 0-8 h after capsule ingestion, but the majority of the metabolites were found in samples collected at 8-24 h. Some metabolites, such as UM6 and UM7, show similar UV spectra characteristics and might therefore be structurally related (Figure 6). In plasma (Figure 5) some metabolites were found in samples collected at 2, 3 and 4 h after capsule ingestion, but the majority of the metabolites were found in samples

collected at later time-points (6-24 h). This could be the result of poor absorption of the high molecular weight phlorotannins in the upper gastrointestinal tract resulting in them reaching the colon and undergoing fermentation to lower molecular weight derivatives by the colonic microbiota. In addition, urine samples were subjected to LC-MS/MS (Figure 7) utilizing electrospray ionisation (ESI) as previously described, searching for the theoretical mono-isotopic masses corresponding to the low molecular weight phlorotannins previously identified in the capsule (Figure 2). We were able to identify molecular ions and fragments corresponding to hydroxytrifuhalol A, 7-hydroxyeckol and the C-O-C dimer of phloroglucinol, which corresponded to the HPLC metabolite UM3. In addition, we reported the presence of 2 ions (289 and 377) corresponding to metabolites that we characterized in samples from SPE subjected to *in vitro* gastrointestinal digestion and fermentation (DM4 and DM7, figure 3) as previously detailed.

***Ex-vivo* Cytokine production:**

The ex-vivo production of cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α) relative to baseline levels in cultured blood collected a various time-points (0, 1, 2, 3, 4, 6 and 8 h) during the intervention study (LPS treated – unstimulated controls) is reported in Fig 8. The amounts of TNF-α and IL-10 remained quite stable over time, as well as the amount of all cytokines at 1h and 2h. IL-6 levels were observed to decrease at later time-points (4 h to 8 h) without reaching statistical significance ($p > 0.05$). The levels of both IL-1β and IL-8 were observed to increase from 3 h to 8 h after the intervention; however the statistical analysis revealed that the only significant change from baseline levels was the increase of IL-8 at 8 h.

Discussion

Polyphenols are ubiquitously found in plants and comprise a major part of a daily human diet. Over the last 20 years, significant data have emerged with regard to the potential beneficial effects of several classes of phenolic compounds against a number of chronic diseases. In addition, a reasonable understanding has been gained of the bioavailability of many polyphenol classes and this will be important for understanding the mechanisms by which they exert such benefits *in vivo*. The interest in marine sources of phenolic compounds is recent and knowledge on phlorotannin bioavailability is still lacking. The analysis of phlorotannins is challenging due to high range of molecular weight present, and their characterisation is complicated further by the lack of commercially available standards.

Chromatography techniques coupled to diode array and MS detection have been applied to the analysis of phlorotannins and the advantages/disadvantages of their use described by Steevensz *et al* [34] . Reversed phase High Performance Liquid Chromatography (RP-HPLC) is a separation mode commonly used for polyphenols separation, however the very high polarity of phlorotannins would causes them to elute with little or no retention due to the lack of interaction with the non-polar stationary phase [34; 48]. Normal phase liquid chromatography (NP-HPLC) is more advantageous for retaining compounds with very high polarity, and the NP-HPLC methodology developed by Koivikko was more suitable than RP-HPLC for the separation of phlorotannins from the brow algae *Fucus vesiculosus* [34; 48]. Thus we initially analysed the phlorotannins in our SPE by Normal-Phase HPLC using a method adapted from Koivikko *et al* [8]. As expected, the SPE comprised a wide range of molecular weights (20-70 min), with abundance of very high molecular weight phlorotannins eluting at later retention time (50-70min) in our Normal-Phase method. MS detection can provide higher sensitivity and be advantageous to identify specific phlorotannins in the extract without commercially available standards, however NP-HPLC solvents such as dichloromethane are not suitable for MS analysis [49], because they would result in poor ionisation and therefore significantly reduce sensitivity [48; 50].

Nwosu *et al*. characterized a phlorotannin extract from *Ascophyllum nodosum* by RP-HPLC using a C18 column the bound sample eluted in a unresolved set of peaks, and with MS detection in negative ion mode they were able to assign the detected m/z spectra to a series of phlorotannin structures [29]. Ferreres *et al*. identified 22 different phlorotannins belonging to the eckol and fucophloroethol groups in four seaweed species belonging to the order Fucales (genus *Cystoseira* and *Fucus*) with RP-HPLC separation combined with DAD-ESI-MSn detection [47]. Using an equivalent RP-HPLC separation method coupled to ESI-MS analysis

384 in negative ion mode we were able to identify some phlorotannin oligomers such as
385 hydroxytrifuhalol A, tetrafucol, fucodiphlorethol, C-O-C dimmer of phloroglucinol, 7-
386 hydroxyeckol, diphloretol and difucol. The fragmentation patterns of the oligomers we
387 identified are in agreement with some recent published data in the field [46; 47]. Recently,
388 Steevensz and his research group characterized the phlorotannins of five brown algae species
389 by ultrahigh-pressure liquid chromatography operating in "mixed-mode" (hydrophilic
390 interaction liquid chromatography mode) combined with high resolution mass spectrometry
391 [34]. The methodology proposed by this research group proved to be accurate for profiling
392 phlorotannins based on their degree of polymerization, and was demonstrated to be an effective
393 separation mode for low molecular weight phlorotannins, up to 6 KDa [34].
394 Phlorotannin characterization is a challenging and complex task, complicated by the lack of
395 commercially available standard compounds, thus chromatography separation coupled to MS
396 detection can help to elucidate phlorotannin complexity and its application to the analysis of
397 clinical samples from feeding trials, as well as the use of simplified *in vitro* digestion systems,
398 can help elucidate their beneficial health properties and the bioactive circulating forms. The
399 SPE was subjected to *in vitro* simulated gastrointestinal digestion and fermentation, followed
400 by dialysis to simulate as close as possible their absorption and biotransformation. The obtained
401 materials were analysed by LC-MS for a comparative characterisation of the phlorotannin
402 metabolites. LC-MA analysis of the digested and fermented SPE have indicated the presence
403 of some oligomeric phlorotannins also present in the undigested SPE (hydroxytrifuhalol A,
404 diphloretol/difucol, 7-hydroxyeckol, C-O-Cdimer of phloroglucinol), in addition to a range of
405 newly formed metabolites (DM1 to DM7). *In vitro* conditions are indeed a great tool, allowing
406 a simpler and more convenient analysis, and our *in vitro* system predicted the formation of
407 metabolites subsequently identified in urine.
408 Intervention studies have investigated the fate of polyphenols from land plants in the human
409 body by measuring plasma concentrations and/or urinary excretion following intake from a
410 food source. Many studies performed to investigate polyphenol bioavailability are based on
411 the measurement of their excretion in urine and plasma by extraction, concentration and
412 chromatographic separation/analysis, and focused on the detection of polyphenols and their
413 metabolites in samples subjected or not to enzymatic treatment to release conjugate moieties
414 such as glucuronic acid and sulphate groups [43; 51]. For example, following ingestion of a
415 polyphenol-rich meal, levels of phenolic compounds and conjugated metabolites can increase
416 rapidly achieving a peak concentration at approximately 1-2 h in plasma and urine, indicating
417 small intestinal absorption, or peak at later time-points (4-8 h in plasma, 8-24 h in urine),

418 indicating large intestinal metabolism and subsequent absorption [52]. In our study, the majority
419 of phlorotannin metabolites were found in samples collected at late time-points (6-24 h),
420 indicating limited small intestinal absorption followed by gut microbial metabolism, of the high
421 molecular weight phlorotannins in the large intestine.
422 In the upper gastrointestinal tract, dietary polyphenols act as substrates for a number of
423 enzymes and are subjected to extensive metabolism by glucosidase enzymes, phase I enzymes
424 (hydrolysing and oxidizing), such as cytochrome P450, and phase II enzymes (conjugating and
425 detoxifying) found both in the small intestine and the liver [37]. In particular there is strong
426 evidence for the extensive phase II metabolism (by UDP-glucuronosyltransferases,
427 sulphotransferases) to yield glucuronides and sulphate derivatives. Indeed, there is evidence of
428 efficient glucuronidation and sulfation of all classes of polyphenols to differing extents [53].
429 Indeed, our results indicate that phlorotannin intake results in the formation of phase II
430 conjugate metabolites (glucuronides, sulfates).
431 Further transformations can occur in the colon, where the enzymes of the gut microbiota act to
432 breakdown complex polyphenolic structures to smaller units, which may also be absorbed and
433 further metabolized in the liver. Bacterial enzymes may catalyse many reactions including
434 hydrolysis, dehydroxylation, demethylation, ring cleavage and decarboxylation as well as rapid
435 de-conjugation [54].
436 As predicted by the high molecular weight range of phlorotannins in our SPE, high colonic
437 metabolism seems to have occurred, following fermentation of high molecular weight
438 phlorotannins in the large intestine. By LC-MS analysis, we were able for the first time to
439 confirm the presence in urine of some phlorotannin oligomers, more specifically
440 hydroxytrifuhalol A, 7-hydroxyeckol and the C-O-C dimer of phloroglucinol. Interestingly, 2
441 of the urine metabolites (*m/z* 289 and 377) were present in the *in vitro* digested samples (DM4
442 and DM7).
443 There were substantial differences between volunteers in the pattern of phlorotannin
444 metabolites. Such inter-individual differences have been observed for other polyphenols and
445 have been attributed to differences in gut microbiota composition and the expression of
446 metabolizing enzymes [3; 55].
447 A secondary aim of our work has been to determine whether the SPE could modulate
448 inflammatory events in the blood, following the absorption of phlorotannin metabolites and
449 due to their presence into the circulation.
450 Polyphenols can exert numerous antioxidant and non-antioxidant functions of relevance in
451 chronic diseases development, and many of them have an important inflammatory component

[50]. In the present study, we observed an altered *ex vivo* production of IL-8, a low-molecular-weight cytokine produced by mononuclear phagocytes and other cell types, with significant increased levels of the cytokine after 8h compared to baseline.

IL-8 is an important inflammatory factor of the CXC chemokine family, involved in the amplification of inflammatory signals [56]. IL-8 secretion is induced by TNF-alfa through a transcriptional mechanism primarily regulated by nuclear factor-kB (NF-kB) [57]. Redox signalling mechanisms are known to play a role in the regulation of such events, with reactive oxygen species being able to promote IL-8 production and secretion [18; 23], whereas oxygen radical scavengers are proven to inhibit IL-8 production in LPS-stimulated human whole blood [22]. Dietary polyphenols such as catechins [33] and curcumin [28] have also been shown to specifically interfere with IL-8 gene expression through inhibition of NF-kB activation [58]. In consequence, we would have expected circulating seaweed polyphenol metabolites to potentially be able to inhibit IL-8 secretion. Our results have given a preliminary indication that the cytokine IL-8 is a possible target for phlorotannin metabolites. However a significant increase on IL-8 levels at 8h after the intervention was observed, in parallel with the presence of phlorotannin metabolites in plasma and urine samples. A recent study from our group investigated the influence of a polyphenol-rich intervention on inflammation as primary outcome. A randomised, double-blind, placebo-controlled, cross-over acute intervention was conducted, and cytokine levels (IL-8) were measured with the same ex-vivo experimental protocol. The results showed a time-dependent increase in IL-8 release compared to baseline, in accordance with our findings. Thus the post-prandial *ex vivo* IL-8 production was significantly attenuated by the intervention compared to the control, with a parallel appearance in the circulation of polyphenol metabolites. Our trial was a single group interventional study primarily designed to investigate the bioavailability of seaweed phlorotannins: however, on the basis of this preliminary indication on their anti-inflammatory potential, not sufficient to draw any conclusion, a chronic placebo-controlled intervention has been conducted to investigate the anti-inflammatory effect in deeper detail.

The main limitations of this study arise from the phlorotannin complexity and lack of commercially available analytical standards, potentially leading to possible quantification inaccuracy as phloroglucinol equivalents. The lack of analytical standards also implies a limited capability for method development, especially for the analysis of plasma, urine and digested materials. In future, the availability of standards could allow a higher degree of method optimization and the development of specific solid phase ion procedures for sample clean-up and concentration.

The development of more recently explored analytical applications to phlorotannins, such as HILIC [32] and NMR [35] could facilitate the development of more suitable protocols that could lead to full identification of metabolites and improvements in phlorotannin metabolites quantification. In addition, The bioacessibility of polyphenols in the form of a capsule/extract might differ greatly from the bioacessibility of the same compounds in a food matrix [59]. Future work will be needed to determine the potential effects on bioavailability of different food matrices and also any effects of cooking and/or processing.

Nevertheless, the present work has for the first time started to shed light on the role of colonic biotransformation on phlorotannin bioavailability, and its implication for their health benefits *in vivo*. Our results provide a basis for further investigating the seaweed-derived bioactive components in the body after ingestion, information which is necessary to understand their mechanism of action *in vivo*.

499 **List of tables:**

500 **Table 1.**

Table 1: Key components of polyphenol rich basic extract, High Molecular Weight (HMW) fraction, and blend (capsule) showing crucial concentrations of polyphenols and iodine.

Extract component	**basic extract** mg/175mg of extract	**HMW fraction** mg/50mg of extract	**blend (capsule)** mg/400mg capsule
Polyphenols	58.74	43.15	101.89
Iodine	0.46	0.02	0.48
Maltodextrin*			175
Minerals	37.77	1.22	38.99
Fucoxanthin	<0.001	0.004	0.004
Laminarin as glucose	10.24	1.64	11.88
Fucoidan as fucose	<0.001	0.23	0.23
Mannitol	28.03	5.53	33.56
Inorganic arsenic	<0.001	<0.001	<0.001
Cadmium (LD 0.15mg/kg)	<LD	<LD	<LD
Mercury (LD 0.016mg.kg)	<LD	<LD	<LD
Lead (LD 1.1mg/kg)	<LD	<LD	<LD
Tin (LD 1.7mg/kg)	<LD	<LD	<LD

*maltodextrin was added to the capsule formulation as an excipient.

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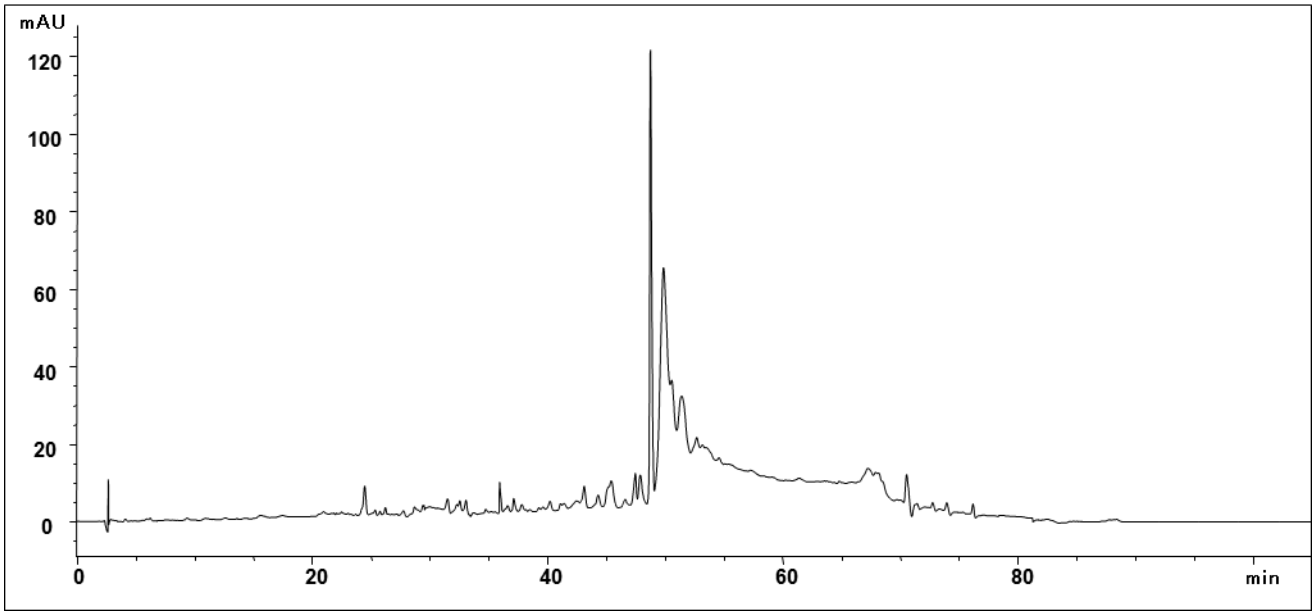
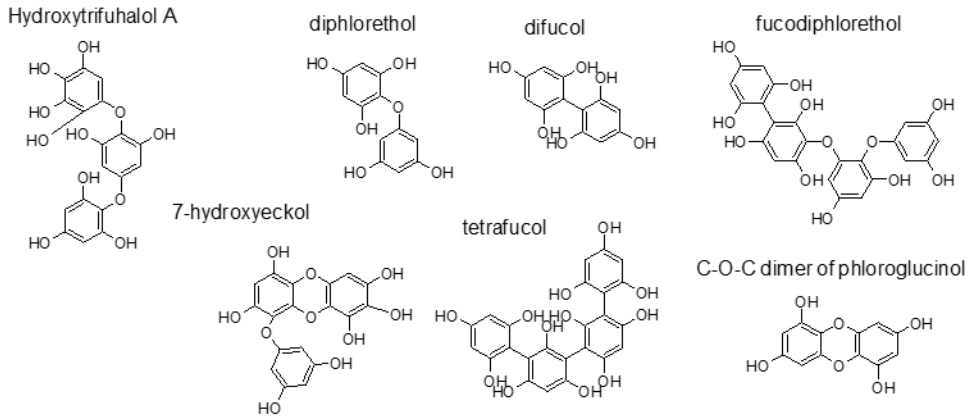
Figure 1



507 **Figure 2**

A



B

LC-MS analysis in negative ion mode of the seaweed extract phlorotannins

Peak N	RT (min)	[M-H]⁻ m/z		MS^2 m/z		tentative identification
1	2.7	405	387	191		hydroxytrifuhalol A
2	3.3	497	479	353	205	tetrafucol, fucodiphlorethol
3	5.6	247	203			C-O-C dimer of phloroglucinol
4	10.6	387	369	230		7-hydroxyeckol
5	43.7	249	181	113		diphlorethol / difucol
6	46.2	249	181	113		diphlorethol / difucol

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510 **Figure 3**

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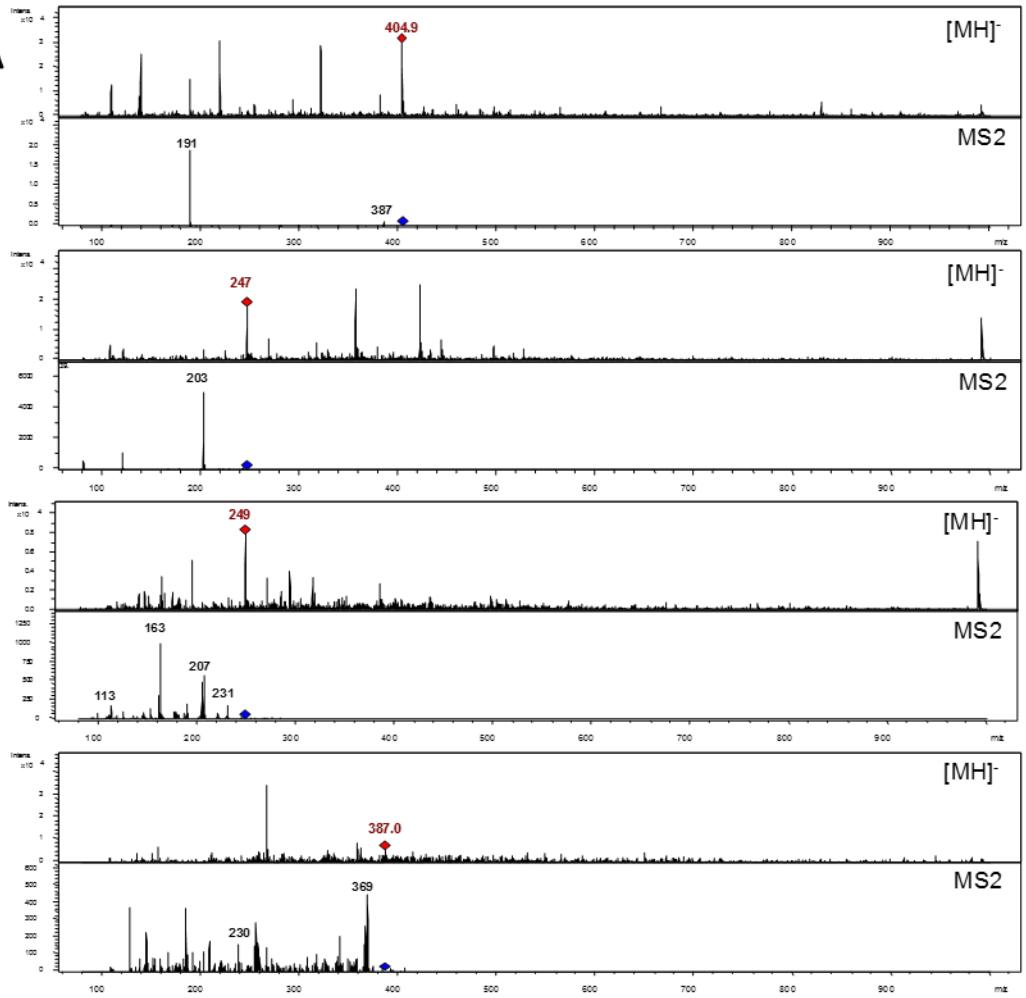

B

LC-MS analysis in negative ion mode of the in vitro digested seaweed extracts

Metabolite	RT (min)	[M-H]- (m/z)	MS2 (m/z)				tentative identification
seaweed extract after small intestinal digestion							
SIM1	2.70	405	387	191			hydroxytrifuhalol A
SIM2	5.00	247	203				C-O-C dimer of phloroglucinol
SIM3	5.70	249	231	207	163	113	diphlorethol / difucol
SIM4	9.30	387	369	230			7-hydroxyeckol
seaweed extract after colonic fermentation							
FM1	9.40	387	369	230			7-hydroxyeckol
dialysed seaweed extract (after digestion and fermentation).							
DM1	1.50	377	311	243	175		Unknown
DM2	1.60	277	211	151	93		Unknown
DM3	1.65	249	181	113			Unknown
DM4	1.70	289	261	221	175		Unknown
DM5	1.75	317	249	225	181	113	Unknown
DM6	1.80	327	269	211	93		Unknown
DM7	1.85	377	341	281	221	179	Unknown

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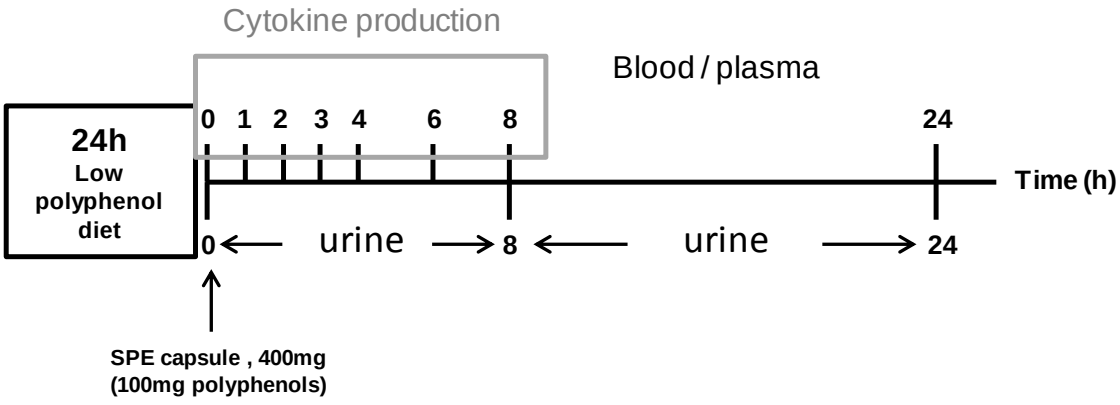
513 **Figure 4**



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516 **Figure 5**

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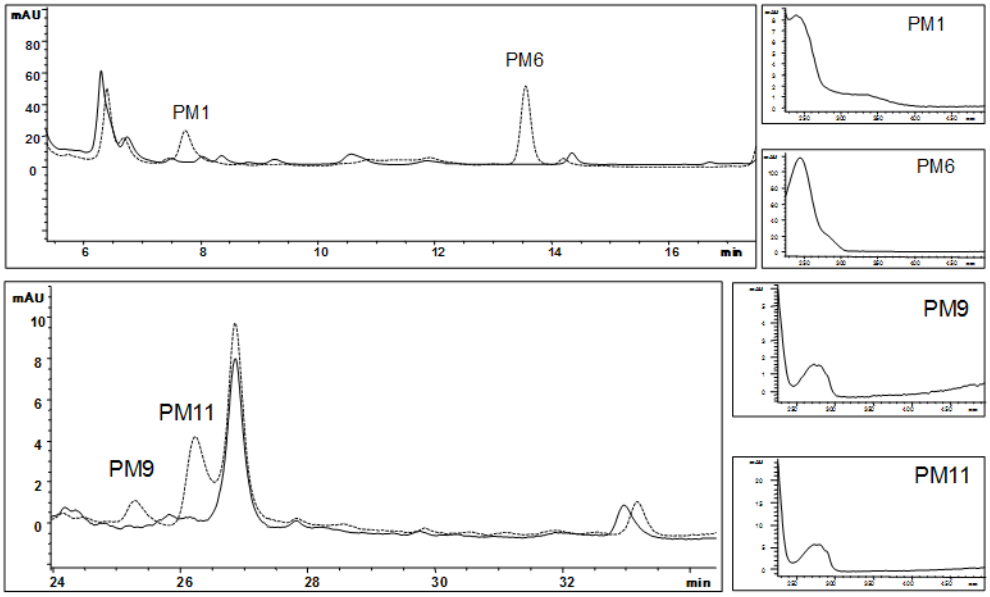

B

RP-HPLC analysis of seaweed metabolites in plasma samples

RT (min)	metabolite	mean (µg/ml)	stdev	N volunteers	time (h)	Enz -	Enz +
6.5	PM1	2.16	2.01	2	4; 24	no	yes
7.7	PM2	0.95		1	24	yes	no
9.2	PM3	0.07		1	8	yes	no
9.3	PM4	0.07	0.03	2	2; 3	yes	no
9.4	PM5	0.05	0.03	7	3; 4; 6; 8; 24	yes	no
13.5	PM6	2.82	1.85	2	24	yes	yes
20.4	PM7	0.03		1	3	no	yes
23.2	PM8	0.03		1	24	no	yes
25.2	PM9	0.06	0.01	2	8; 24	yes	no
25.5	PM10	0.01	0.00	2	24	yes	yes
26.2	PM11	0.22		1	24	yes	no
30.3	PM12	0.41		1	24	yes	no
32.7	PM13	0.05	0.04	3	24	yes	yes
33.2	PM14	0.04		1	4	yes	no
36.1	PM15	0.01		1	2	yes	no
38.0	PM16	0.02	0.01	3	2; 3	yes	no
42.4	PM17	0.04		1	4	yes	no
44.7	PM18	0.03	0.03	5	6; 24	yes	yes

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520 **Figure 6**

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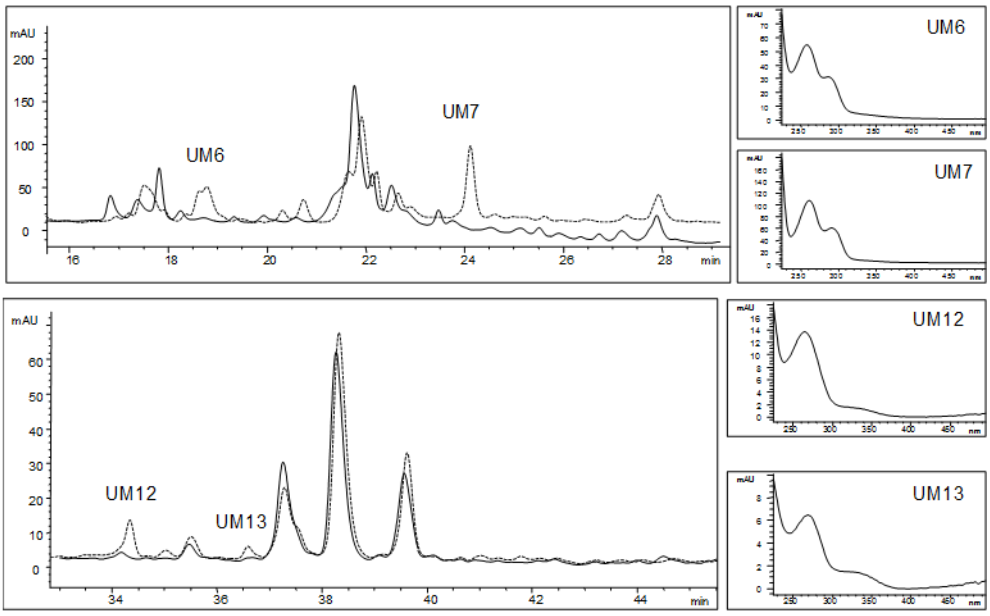

B

RP-HPLC analysis of seaweed metabolites in urine samples

RT (min)	metabolite	mean (mg)	stdev	N volunteers	time (h)	Enz -	Enz +
7.9	UM1	15.25	1.39	2	0 -- 8; 8 -- 24	yes	yes
10.4	UM2	10.77	4.29	7	8 -- 24	yes	yes
14.2	UM3	16.66	0.89	2	8 -- 24	yes	yes
16.7	UM4	2.94	1.82	3	8 -- 24	yes	yes
17.7	UM5	0.67		1	8 -- 24	yes	no
18.7	UM6	5.58	0.17	2	8 -- 24	yes	yes
24.1	UM7	9.85		1	8 -- 24	no	yes
29.6	UM8	0.30		1	8 -- 24	yes	no
30.8	UM9	0.51		1	8 -- 24	yes	no
33.0	UM10	1.26		1	8 -- 24	no	yes
33.1	UM11	1.28	0.09	2	8 -- 24	yes	yes
34.3	UM12	0.63	0.15	2	8 -- 24	yes	yes
36.5	UM13	0.61	0.55	6	8 -- 24	yes	yes
41.8	UM14	1.45	0.86	8	0 -- 8; 8 -- 24	no	yes
43.7	UM15	0.25		1	8 -- 24	no	yes
45.5	UM16	0.69		1	8 -- 24	yes	no
46.1	UM17	0.25		1	8 -- 24	no	yes
46.7	UM18	0.70		1	8 -- 24	no	yes

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523 **Figure 7**

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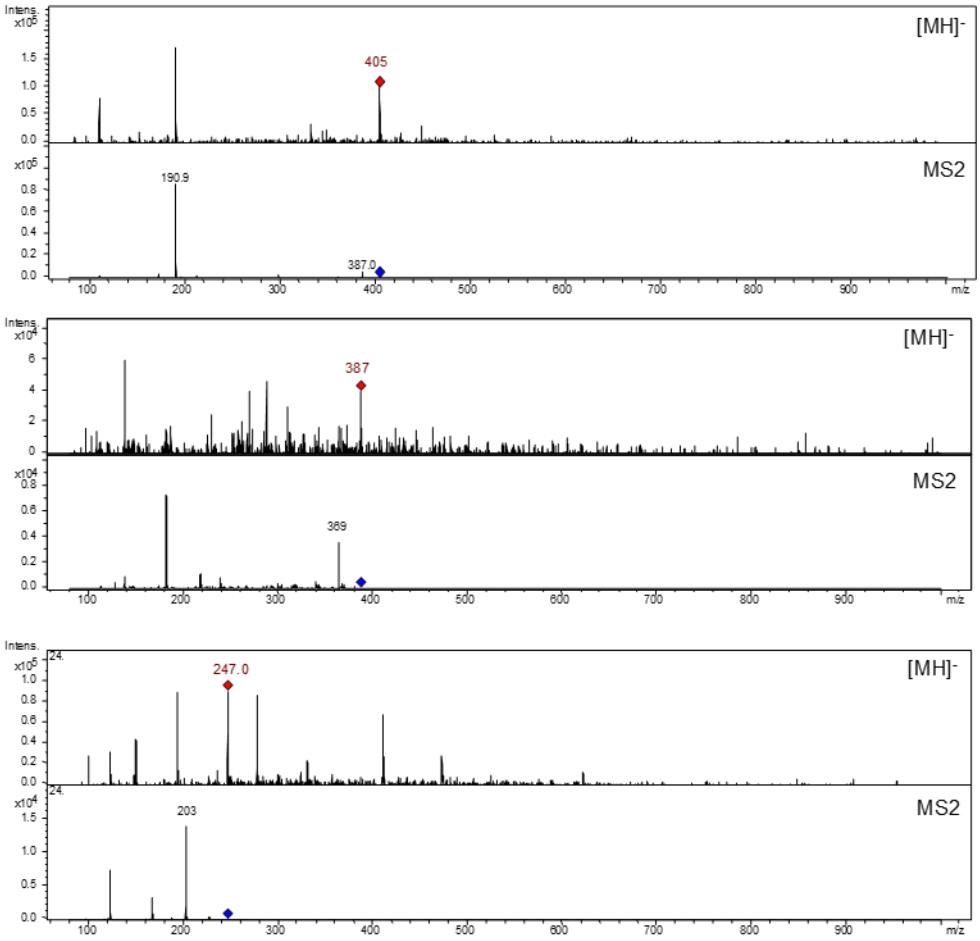

B

LC-MS analysis in negative ion mode of the urine samples

RT (min)	[M-H]- (m/z)	MS2 (m/z)		tentative identification
2.20	405	387	191	hydroxytrifuhalol A
4.10	387	369		7-hydroxyeckol
10.40	247	203		C-O-C dimer of phloroglucinol , UMA3
1.70	289	221	175	Unknown, DM4
1.85	377	341	161	Unknown, DM7

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526 **Figure 8**

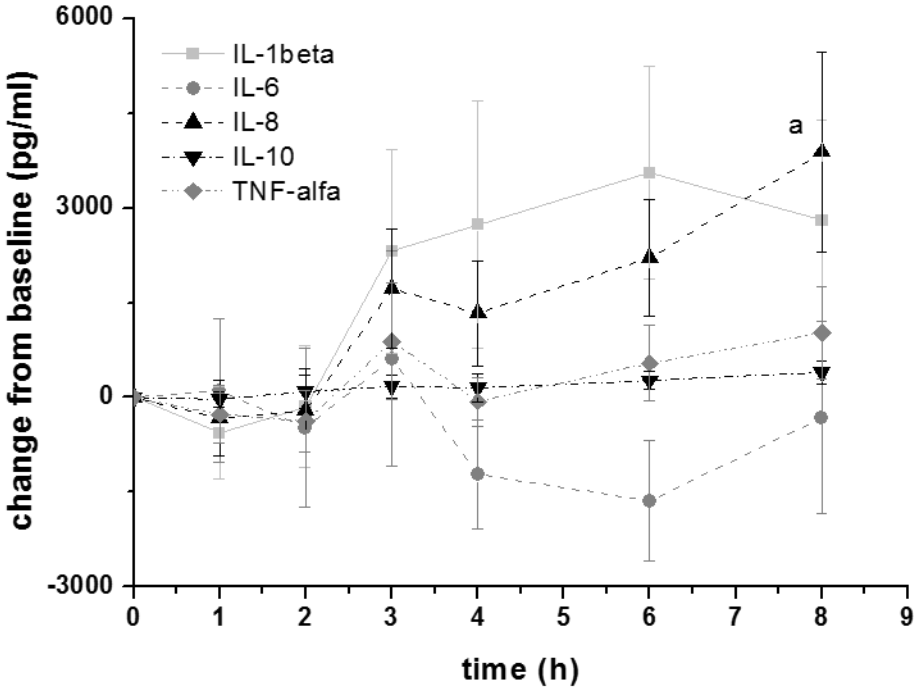

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FIGURE LEGENDS

Figure 1. Chromatographic separation of phlorotannins contained in the seaweed extract by Normal-Phase HPLC with diode array detection (268 nm).

Figure 2. Characterisation of phlorotannins in the seaweed extract. A: Structures of phlorotannins identified in the seaweed extract: B: Phlorotannins in the seaweed extracts identified by LC-MS analysis in negative ion mode

Figure 3. LC-MS analysis in negative ion mode of the seaweed extract subjected to *in vitro* gastrointestinal digestion, colonic fermentation and dialysis to mimic absorption. A: LC-MS spectra and fragmentation of *in vitro* digested materials. B: Summary of LC-MS analysis of the *in vitro* digested materials

Figure 4. Schematic illustration of the clinical intervention set up.

Figure 5. HPLC analysis of plasma samples for seaweed metabolites. A: HPLC chromatograms (268 nm) and UV spectras showing examples of metabolites in plasma. B: summary of seaweed metabolites present in plasma samples.

Figure 6. HPLC analysis of urine samples for seaweed metabolites. A: HPLC chromatograms (268 nm) and UV spectras showing examples of metabolites in urine. B: summary of seaweed metabolites present in urine samples.

Figure 7. LC-MS analysis in negative ion mode of urine samples. A: LC-MS spectra and fragmentation of phlorotannins found in urine samples. B: Summary of LC-MS analysis of the urine samples.

Figure 8. Cytokine production by whole blood cultures in cultured blood collected a various time-points (0, 1, 2, 3, 4, 6 and 8 h) during the intervention study (LPS treated – unstimulated controls). a = $P < 0.05$ vs. baseline.

Financial support

This work is part of the SWAFAX project (no. 262519), funded by the European Commission under the Capacities Programme (FP7)

Conflict of interest

None

Authorship

G.C., J.P.E.S, P.Y. and I.R. designed the research. G.C. organized and coordinated all parts of the clinical trial and analytical work. S.H. overviewed the preparation of seaweed materials, extracts and capsules. P.A. aided running the clinical trial. Y.J. aided with the analysis of metabolites in plasma and urines. G.C. analysed and summarised all the data. G.C. drafted the manuscript, C.G., I.R, S.H. and P.Y. revised the manuscript. I.R. had primary responsibility for final content.

LIST OF ABBREVIATIONS

SPE Seaweed Polyphenol Extract

DM Digestion Metabolite

UM Urine Metabolite

PM Plasma Metabolite

ESI electrospray ionization

RP-HPLC Reverse Phase High Performance Liquid Chromatography

NP-HPLC Normal Phase Liquid Chromatography

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